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Schoch, H J

Abstract: Formation of cerebral oedema caused by vascular leakage is a major problem in various injuries of the CNS, such as stroke, head injury and high-altitude illness. A common feature of all these disorders is the fact that they are associated with tissue hypoxia. Hypoxia has therefore been suggested to be an important pathogenic factor for the induction of vascular leakage in the brain. Vascular endothelial growth factor (VEGF) is known as the major inducer of angiogenesis. Originally, however, it was described as a vascular permeability factor. As VEGF gene expression was shown to be upregulated by hypoxia, increased VEGF expression may link hypoxia and vascular leakage in the CNS in vivo. To delineate the role of VEGF in vascular leakage in the brain, we studied the effect of hypoxia on VEGF expression and vascular permeability in the brains of mice in vivo. Hypoxic exposure led to a significant increase in the levels of VEGF mRNA and protein in mouse brain that correlated with the severity of the hypoxic stimulus. Measurement of vascular permeability using the fluorescent marker sodium fluorescein revealed a two-fold increase in fluorescence intensity in hypoxic brains, indicative of significant vascular leakage. Inhibition of VEGF activity by a neutralizing antibody completely blocked the hypoxia-induced increase in vascular permeability. In conclusion, our data show that VEGF is responsible for hypoxia-induced augmentation in vascular leakage following tissue hypoxia. Our findings might provide the basis for new therapeutic concepts for the treatment of cerebral oedema

DOI: https://doi.org/10.1093/brain/awf257

Brain (2002), **125,** 2549–2557

Hypoxia-induced vascular endothelial growth factor expression causes vascular leakage in the brain

Heike J. Schoch,[1] Silvia Fischer[2] and Hugo H. Marti[1,3]

[1]*Department of Molecular Cell Biology, Max Planck Institute for Physiological and Clinical Research,* [2]*Department of Anesthesiology and Intensive Care, Kerckhoff Clinic, Bad Nauheim, Germany and* [3]*Institute of Physiology, University of Zürich, Zürich, Switzerland*

Correspondence to: PD Dr med Hugo H. Marti, University of Zürich, Institute of Physiology, Winterthurerstrasse 190, CH-8057 Zürich, Switzerland
E-mail: marti@physiol.unizh.ch

Summary

Formation of cerebral oedema caused by vascular leakage is a major problem in various injuries of the CNS, such as stroke, head injury and high-altitude illness. A common feature of all these disorders is the fact that they are associated with tissue hypoxia. Hypoxia has therefore been suggested to be an important pathogenic factor for the induction of vascular leakage in the brain. Vascular endothelial growth factor (VEGF) is known as the major inducer of angiogenesis. Originally, however, it was described as a vascular permeability factor. As VEGF gene expression was shown to be up-regulated by hypoxia, increased VEGF expression may link hypoxia and vascular leakage in the CNS *in vivo*. To delineate the role of VEGF in vascular leakage in the brain, we studied the effect of hypoxia on VEGF expression and vascular permeability in the brains of mice *in vivo*. Hypoxic exposure led to a significant increase in the levels of VEGF mRNA and protein in mouse brain that correlated with the severity of the hypoxic stimulus. Measurement of vascular permeability using the fluorescent marker sodium fluorescein revealed a two-fold increase in fluorescence intensity in hypoxic brains, indicative of significant vascular leakage. Inhibition of VEGF activity by a neutralizing antibody completely blocked the hypoxia-induced increase in vascular permeability. In conclusion, our data show that VEGF is responsible for hypoxia-induced augmentation in vascular leakage following tissue hypoxia. Our findings might provide the basis for new therapeutic concepts for the treatment of cerebral oedema.

Keywords: vascular permeability; oedema; VEGF; HACE; ischaemia

Abbreviations: BBB = blood–brain barrier; HACE = high-altitude cerebral oedema; HIF = hypoxia-inducible factor; r.f.u. = relative fluorescence unit; VEGF = vascular endothelial growth factor

Introduction

Oedema formation is a major life-threatening complication of various injuries of the CNS, such as head injury (Murakami *et al*., 1999; Pilitsis and Rengachary, 2001), tumour growth (Papadopoulos *et al*., 2001) and cerebral ischaemia (Lipton, 1999; Rosenberg, 1999). It also occurs during high-altitude illness (Hackett and Roach, 2001). Acute mountain sickness and high-altitude cerebral oedema (HACE) refer to cerebral abnormalities of high-altitude illness and are syndromes that occur in unacclimatized persons on ascent to high altitude (Hackett, 1999). Acute mountain sickness is characterized by the presence of headache, nausea, insomnia, dizziness and fatigue (Hackett and Roach, 2001) and is probably due to the formation of mild cerebral oedema (Hackett, 1999). HACE, clinically defined as the onset of ataxia and altered consciousness, has been considered to be the end-stage of acute mountain sickness, eventually leading to death caused by brain herniation (Hackett and Roach, 2001). While the clinical aspects of HACE are well established, its pathophysiology remains elusive. Recent evidence suggests that HACE is associated with osmotic cell swelling, vasogenic oedema and biochemical alteration of the blood–brain barrier (BBB) (Severinghaus, 1995; Hackett, 1999; Hackett and Roach, 2001). Vasogenic brain oedema is defined as the translocation of proteins and fluid from the vascular space across the BBB (Hackett, 1999). The underlying molecular and pathogenic mechanisms are poorly understood. Several years ago, Severinghaus and Xu hypothesized that the vascular endothelial growth factor (VEGF) might be respon-

sible for the vascular leakage and oedema formation in the brain that occurs during high-altitude exposure (Severinghaus, 1995; Xu and Severinghaus, 1998). VEGF is the most prominent member of an increasing family of angiogenic growth factors and plays a key role in new vessel growth. VEGF is an endothelial mitogen and exists as several isoforms derived from a single gene by alternative splicing (Robinson and Singer, 2001). It is ubiquitously expressed, binds to two endothelial tyrosine receptors, VEGF receptor 1 (VEGFR-1, Flt-1) and VEGFR-2 (Flk-1/KDR), and is also a ligand for the semaphorin receptors neuropilin 1 and neuropilin 2 (Robinson and Singer, 2001).

There is good evidence to support the hypothesis of Severinghaus and Xu. First, all the above-mentioned brain pathologies involving oedema formation are associated with tissue hypoxia. For example, hypoxic regions have been identified around brain tumours (Shweiki *et al*., 1992; Evans *et al*., 1997; Damert *et al*., 1997; Talks *et al*., 2000). Also, during cerebral ischaemia, a region around the core of the infarct—the so-called penumbra—suffers from hypoxia (Evans *et al*., 1997; Read *et al*., 1998; Marti *et al*., 2000). Finally, at high altitude, reduced ambient pO_2 levels result in systemic tissue hypoxia that also affects the brain. Thus, tissue hypoxia could play an important role in the pathogenesis of vascular leakage leading to brain oedema formation, e.g. by activating specific genes. Secondly, *VEGF* is among the best-known of the genes that are induced by hypoxia (Shweiki *et al*., 1992; Marti and Risau, 1998). VEGF gene expression is known to be activated under hypoxic conditions at the level of transcription, increased stability of the mRNA and preferential translation (Ikeda *et al*., 1995; Levy *et al*., 1995; Stein *et al*., 1998). Transcriptional activation is achieved by the transcription factors hypoxia-inducible factor 1 (HIF-1) and HIF-2 (Forsythe *et al*., 1996; Ema *et al*., 1997). HIF-1 binds to a hypoxia response element (HRE) in the 5′-flanking region of the *VEGF* gene, thereby enhancing transcription of the gene (Forsythe *et al*., 1996). Increased VEGF levels in hypoxic tissues are thought to induce an angiogenic reaction that enables increased delivery of nutrients and oxygen to the hypoxic cells through newly formed vessels (Shweiki *et al*., 1992; Marti and Risau, 1999). Tissue hypoxia and angiogenesis are thus interdependent, and have indeed been observed to coincide during cerebral ischaemia (Marti *et al*., 2000), tumour growth (Damert *et al*., 1997) and chronic hypoxic situations (Harik *et al*., 1995; LaManna and Harik, 1997). Thirdly, VEGF is expressed in the CNS in astrocytes and, after hypoxic exposure, in neurones (Marti and Risau, 1998; Ogunshola *et al*., 2000). Finally, VEGF was originally isolated as a factor leading to increased vascular permeability in tumours and was named vascular permeability factor (Senger *et al*., 1983).

Thus, VEGF might be a significant contributor to hypoxia-induced vascular leakage and oedema formation in the brain (Xu and Severinghaus, 1998). If so, inhibition of VEGF action could prevent brain oedema formation and thus be useful as a therapeutic strategy to treat patients with HACE and other diseases associated with oedema formation. We therefore analysed vascular leakage and expression of VEGF during systemic normobaric hypoxia in the brains of mice *in vivo* and inhibited VEGF action by systemic application of a neutralizing anti-VEGF antibody.

Material and methods

Animals

All experiments were performed according to protocols approved by the Regierungspräsidium Darmstadt. Adult C57/BL6 mice were exposed to normobaric hypoxia at 12–6% oxygen (corresponding to an altitude of 4000–9500 m) for 24 h or were kept at room air pressure. Hypoxia was achieved by substituting nitrogen for oxygen using a Digamix 5SA 18/3a pump (H. Wösthoff, Bochum, Germany) after gradual adaptation over a period of 1 h (Marti and Risau, 1998). Mice had free access to food and water. Following hypoxic exposure, the animals were killed by decapitation and the dissected brains were frozen in liquid nitrogen or embedded for cryosectioning.

Determination of brain water content and vascular permeability

To quantify brain water content in brains from normoxic and hypoxic animals, the wet weights of the brains were determined immediately after removal. The samples were dried at 110°C for 24 h and reweighed to give the dry weight. The difference between the wet and dry weights was taken as the *in vivo* water content.

To quantify vascular permeability of brain vessels, 200 µl of sodium fluorescein (Sigma, Taufkirchen, Germany) at a concentration of 6 mg/ml in PBS (phosphate-buffered saline) was injected through the tail vein after 24 h of hypoxic or normoxic exposure. Sodium fluorescein (MW 376.3) is a fluorescent tracer that does not cross an intact BBB (Williams *et al*., 1984). Thirty minutes later, mice were anaesthetized and then perfused with PBS (20 ml) through the left heart ventricle to remove the fluorescent tracer from the vascular bed. Subsequently, both hemispheres of the brain were removed and frozen in liquid nitrogen. To assess fluorescence, brain hemispheres were homogenized in 0.5 M borate buffer (pH 10) and centrifuged (3000 r.p.m.) for 15 min at 4°C, and the supernatant was added to 1.2 ml of ethanol to precipitate proteins. Probes were again centrifuged (13 000 r.p.m.) for 20 min at 4°C and the fluorescence of the supernatant was measured at 485 nm at an excitation wavelength of 330 nm using a Lambda Fluoro 320 Fluoroscope (MWG Biotech, Ebersberg, Germany) (Baba *et al*., 1988). Results are presented as relative fluorescence units (r.f.u.) per mg of brain tissue. In a second set of experiments, a neutralizing goat anti-mouse VEGF antibody or a corresponding control goat IgG antibody (R&D Systems, Minneapolis, MN, USA) was injected intraperitoneally at

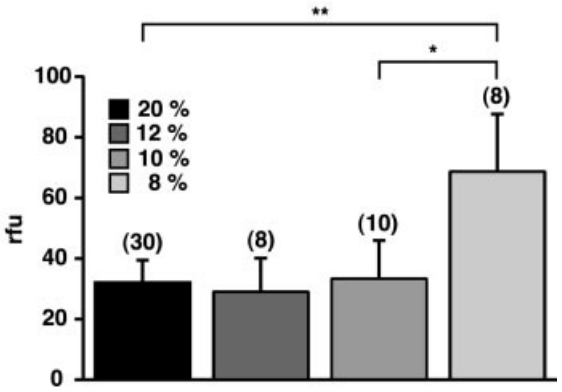

Fig. 1 Two-fold increase in vascular permeability after exposure to 8% oxygen. Sodium fluorescein injected intravenously in controls or hypoxic mice was quantified following homogenization of brain hemispheres. Results are expressed as relative fluorescence units (r.f.u.). Values are mean and standard deviation. $^{**}P < 0.0001$; $^{*}P < 0.001$; $n = 8$–30 as indicated.

100 µg (dissolved in 100 µl PBS) immediately prior to the 24 h hypoxic or normoxic exposure.

RNA extraction, Northern blot analysis and in situ *hybridization*

Total RNA was extracted using peqGOLD RNA pure (Peqlab Biotechnologie, Weingarten, Germany) essentially as described (Wenger *et al*., 1996). Northern blot analysis was performed as described (Marti *et al*., 1996; Wenger *et al*., 1996). Hybridization patterns with RNA for the ribosomal protein L28, which remains unaffected by hypoxia (Wenger *et al*., 1995), were used to correct for loading differences of total RNA on each lane. To quantify VEGF levels, the relative intensities for the hybridization signal (VEGF/L28) were calculated and are presented as percentages of the normoxic control. VEGF *in situ* hybridization was performed exactly as described (Breier *et al*., 1992; Marti and Risau, 1998).

Mouse VEGF immunoassay

Cortical brain tissue lysates from normoxic and hypoxic animals were prepared as described (Marti *et al*., 2000). Mouse VEGF was quantified using a commercially available immunoassay kit (Quantikine M; R&D Systems) as described (Marti *et al*., 2000).

Presentation of data and statistics

Steady-state mRNA levels for VEGF and L28 were recorded and quantified using a Bio-Imaging Analyzer (BAS-2500; Fuji, Raytest GmbH, Straubenhardt, Germany). VEGF signals were then normalized to the signal obtained with the L28 cDNA probe to correct for loading and blotting differences. For hypoxia-inducible expression of VEGF, mRNA and protein levels were expressed as percentages of the corresponding normoxic control. For the statistical analysis of the data, means and standard deviations of n experiments were determined and Student's t-test was performed.

Results

Normobaric hypoxia induces vascular leakage in the brain

To mimic a stay at high altitude, mice were exposed to various fractions of inspiratory oxygen in a normobaric hypoxic chamber for a period of 24 h. To explore whether exposure to severe hypoxia led to the occurrence of HACE, we first examined changes in brain water content as a sign of oedema formation in this organ. Brain water content increased slightly, but not significantly, from 77.99 ± 0.31% (n = 11) in normoxic controls to 78.92 ± 1.96 (n = 12) after hypoxic exposure. The small increase observed might have been due to considerable water loss resulting from severe hyperventilation, as animals that were exposed for 24 h to hypoxia lost more than 10% of their body weight, whereas the normoxic control mice kept their weight constant.

To find out directly whether vascular leakage had occurred as a result of hypoxic exposure, we quantified vascular permeability by a direct method. Sodium fluorescein was injected through the tail vein and allowed to circulate. Sodium fluorescein is a fluorescence tracer that can be measured easily in homogenates of brain hemispheres to exactly quantify dye extravasation and thus vascular permeability.

Relative fluorescence intensity in the brain of mice exposed to 8% oxygen increased more than two-fold ($P < 0.0001$) compared with normoxic controls, indicative of increased vascular leakage of the dye (Fig. 1). Fluorescence in control brain hemispheres was 31.38 ± 4.59 r.f.u./mg brain tissue (n = 30) and increased to 68.70 ± 18.97 r.f.u./mg (n = 8) at 8% oxygen. To address the question whether there is a threshold level above which vascular permeability is unaffected, we exposed mice in addition to 12 and 10% oxygen. Under both conditions, dye extravasation was not significantly different from that in normoxic control animals (12% oxygen, 29.04 ± 11.12 r.f.u./mg, n = 8; 10% oxygen, 33.38 ± 12.58 r.f.u./mg, n = 10). Thus, vascular leakage of fluorescent dye during severe hypoxic exposure suggests that during exposure to 8% oxygen a significant increase in vascular permeability of cerebral microvessels occurred. Furthermore, there was a sharp and significant increase in vascular permeability when animals were exposed to 8% oxygen compared with exposure to 10% ($P < 0.001$)

Increased VEGF gene expression in the brain is dependent on severity of hypoxia

An important question concerns the mechanisms that may link tissue hypoxia and increased vascular permeability. A promising candidate is the angiogenic growth factor VEGF.

Expression of VEGF mRNA and protein levels in normoxic and hypoxic brains was therefore analysed using Northern blotting, *in situ* hybridization and ELISA (enzyme-linked immunosorbent assay) techniques. VEGF mRNA was detectable by Northern blot analysis in all brain samples tested (Fig. 2, upper panel). In normoxic brains, though, mRNA levels were very low, but increased slightly after exposure to 12% oxygen. A significant increase was seen at 10% oxygen, and mRNA levels rose further at 8 and 6% oxygen (Fig. 2, lower panel). VEGF mRNA levels did not differ significantly between 6 and 8% oxygen.

To analyse the regional distribution of VEGF expression under normoxic and hypoxic conditions, *in situ* hybridization experiments were performed. In normoxic controls, VEGF mRNA expression was very low and was homogeneous throughout the brain (Fig. 3). Stronger expression was detected only in the epithelial cells of the choroid plexus, as described previously (Marti and Risau, 1998). After hypoxic exposure, levels of VEGF mRNA were clearly increased all over the cortex, in the hippocampus, cerebellum and the subventricular zone, whereas expression was diminished in the choroid plexus. Thus, with the exception of epithelial cells of the choroid plexus, exposure to hypoxic hypoxia led to general activation of VEGF gene expression in the whole brain. Taken together, our results demonstrate a strong, continuous, oxygen-regulated increase in VEGF gene expression throughout the brain.

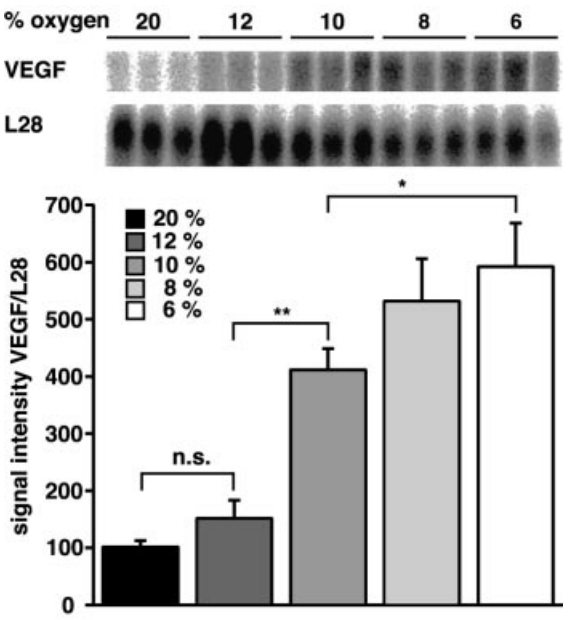

Fig. 2 Increased expression of VEGF mRNA in mouse brain after hypoxic stimulation. Total RNA was extracted from brains of normal mice and mice exposed to 6–12% oxygen for 24 h. (*Upper panel*) Northern blots of total RNA sequentially hybridized with a ^{32}P-labelled probe for murine VEGF and the ribosomal protein L28. (*Lower panel*) Mean and standard deviation ($n = 3$) of VEGF mRNA pixel densities as quantified with a Phosphoimager and corrected for L28. Normoxic control was set to 100%. $^{**}P < 0.001$; $^{*}P < 0.05$; n.s., not significant.

To confirm these mRNA data at the protein level, we isolated total cortical proteins and quantified the amount of VEGF protein present by ELISA. The basal normoxic level of VEGF was 2.4 ± 0.9 ng/g total protein, which increased to 9.6 ± 1.8 and 11.6 ± 5.0 ng/g after exposure to 12 and 10% oxygen, respectively. A significant further increase was found at 8% (19.5 ± 2.3 ng/g total protein) and 6% (21.9 ± 3.6 ng/g total protein) oxygen ($P < 0.05$) (Fig. 4). Again, the VEGF level did not differ significantly between 6 and 8% oxygen. Thus, both RNA and protein data confirm a continuous increase in VEGF production in the brain when systemic oxygen availability is decreased progressively. These results implicate VEGF in the generation of vascular leakage and brain oedema during episodes of severe tissue hypoxia.

Blocking VEGF action prevents vascular permeability

To further test the hypothesis that VEGF confers hypoxia-related leakage of cerebral blood vessels and may thus be responsible for the occurrence of HACE, neutralizing antibodies directed against mouse VEGF were applied immediately before hypoxic exposure by injecting 100 μg of a neutralizing goat anti-mouse VEGF antibody intraperitoneally. An isotypic goat IgG antibody was used as a specific negative control. As expected from the first round of experiments, vascular permeability increased after hypoxic exposure to 8% oxygen, i.e. the r.f.u. level rose from 30.69 ± 7.16 ($n = 4$) in normoxic controls to 58.60 ± 15.63 per mg brain tissue ($n = 7$) in hypoxic animals (Fig. 5). In sharp contrast, the hypoxic increase in vascular permeability in mice treated with VEGF antibody was completely prevented (29.59 ± 3.92 r.f.u./mg, $n = 4$), demonstrating the causative relationship between hypoxic VEGF induction and hypoxia-induced vascular leakage in the brain.

Discussion

This study demonstrates that inhibition of VEGF action is able to prevent hypoxia-induced vascular leakage in the brain. Our data support the hypothesis that exposure to systemic hypoxia activates VEGF gene transcription in the CNS, leading to increased VEGF protein levels, which in turn increase vascular permeability in brain microvessels. Furthermore, our results demonstrate the existence of a threshold level at ~10% oxygen at which VEGF expression starts to increase but no change in vascular permeability is yet observed. Our findings provide the basis for the treatment of oedema formation in the CNS that is associated with brain injury, stroke and HACE.

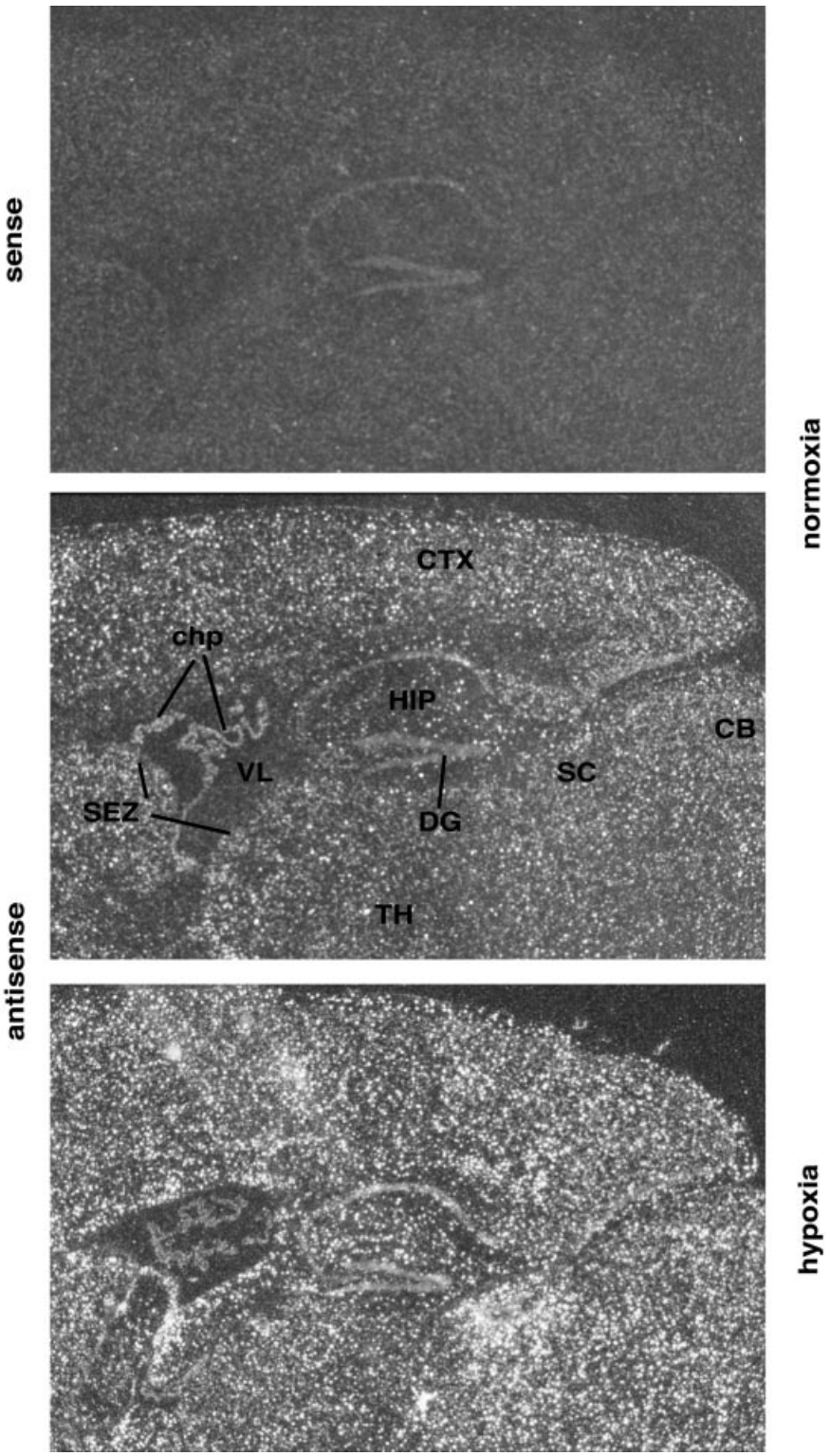

Fig. 3 VEGF mRNA expression detected by *in situ* hybridization on sagittal sections of mouse brain. Following exposure to 8% oxygen (*lower panel*), VEGF mRNA is upregulated homogeneously all over the brain compared with normoxic controls (*middle panel*). (*Upper panel*) Hybridization with a VEGF sense probe. CB = cerebellum; chp = choroid plexus; CTX = cerebral cortex; DG = dentate gyrus; HIP = hippocampal region; SC = superior colliculus; SEZ = subependymal zone; TH = thalamus; VL = lateral ventricle.

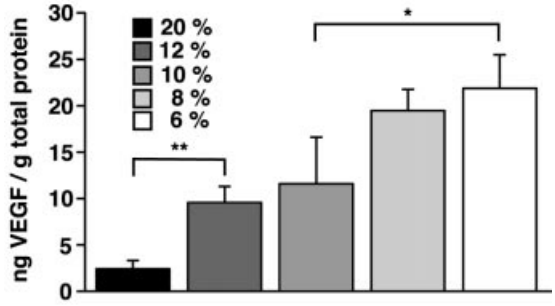

Fig. 4 Increased VEGF protein levels in the hypoxic brain. The amount of VEGF protein present in the brain was measured using an ELISA (enzyme-linked immunosorbent assay) specific for murine VEGF. Values are mean and standard deviation ($n = 3$). $^{**}P < 0.01$; $^{*}P < 0.05$. Note the inverse correlation between VEGF protein level and oxygen concentration.

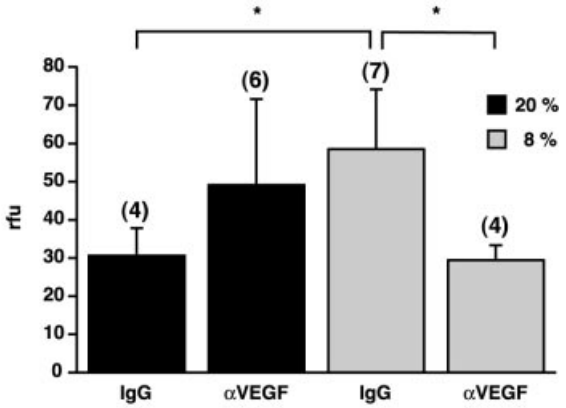

Fig. 5 Blocking VEGF action prevents hypoxia-induced vascular leakage in the brain. A neutralizing anti-VEGF antibody (αVEGF) or an isotypic goat antibody (IgG) was injected intraperitoneally before exposure to 20% (black columns) or 8% oxygen (grey columns) for 24 h. Vascular permeability was assessed as described in Figure 2. Values are mean and standard deviation. $^{*}P < 0.01$; $n = 4$–7 as indicated.

Inverse correlation between VEGF expression and inspired oxygen concentration

We noted that VEGF expression started to increase at ~10% oxygen, but only after exposure to 8% oxygen were significant changes in vascular permeability in the brain microvessels observed, suggestive of the existence of defined threshold levels for VEGF expression and permeability changes. Activation of VEGF expression during hypoxic exposure has been investigated in a number of studies, although these investigated mostly the effects of exposure time, not variation in oxygen concentration (Patt *et al*., 1998; Xu and Severinghaus, 1998; Kuo *et al*., 1999). Increased VEGF gene expression is thus found consistently when inspired oxygen levels fall below 10%, which corresponds to the amount of oxygen present at ~5300 m altitude. In our mouse model, vascular leakage was detected only after 24 h of exposure to 8% oxygen, corresponding to 7100 m altitude. The occurrence of HACE in humans is increasingly common and severe at altitudes higher than 4000 m, while the incidence is clearly reduced at 3000 m (Severinghaus. 1995). Thus, also in humans there is a clear relation between the occurrence of HACE symptoms and the degree of hypoxia. The difference in the degree of hypoxia required to elicit vascular leakage and oedema formation in humans and mice might be explained by the fact that in humans it is mostly clinical symptoms that are considered (Hackett and Roach, 2001), whereas in our mouse model we sought direct proof of brain vascular leakage. It might also be due to species differences, since it has been shown that small animals have a higher capillary density in the muscles compared with larger species (Schmidt-Nielsen and Pennycuik, 1961). This may also hold true for the brain, resulting in a shorter diffusion distance from the capillary to the metabolizing cell, thus making mice more resistant to hypoxia than man.

VEGF gene expression is transcriptionally regulated by HIF-1 (Forsythe *et al*., 1996). We have previously demonstrated *in vitro* that HIF-1 activation is inversely related to oxygen concentration, with a half-maximal activation of HIF at 1.5–2% oxygen (Jiang *et al*., 1996). Although we did not measure pO_2 within the brain parenchyma of our mice, data from the literature suggest that normal tissue pO_2 levels in the brain are in the range of 20 mm Hg (2.5% oxygen) (Whalen *et al*., 1970). During hypoxic exposure, levels certainly drop further, to a range where even small changes in pO_2 lead to strong activation of HIF-1. Indeed, it was shown very recently that HIF-1 protein accumulated gradually in the brain within 2–6 h when mice were exposed to decreasing amounts of oxygen ranging from 21 to 6% (Stroka *et al*., 2001). Thus, activation of HIF-1 precedes upregulation of VEGF expression, which will then lead to increased permeability, given a strong enough hypoxic stimulus.

A clinically important question which arises from our findings that different hypoxic threshold levels exist for the activation of VEGF gene expression and the induction of vascular leakage is whether VEGF levels can be used as a marker to identify patients at risk of developing HACE. As it is not feasible to analyse brain VEGF levels in humans, one might look at plasma VEGF levels. In the plasma of our hypoxic mice ($n = 3$), we found that VEGF levels were elevated less than two-fold and non-significantly at both 12 and 8% oxygen (3.9 ± 0.63 and 3.7 ± 0.57 ng VEGF/ml, respectively) compared with normoxic controls (2.4 ± 0.27 ng VEGF/ml). This result is in good agreement with a study in humans demonstrating no increase in plasma VEGF at 4200 m and no correlation of VEGF with oxygen saturation or symptoms of acute mountain sickness (Maloney *et al*., 2000). Furthermore, in a recent study investigating plasma VEGF levels in humans at high altitude (4559 m), no correlation between VEGF and the occurrence of acute mountain sickness symptoms was found either, although VEGF in the plasma was significantly increased after hypoxic exposure (Walter *et al*., 2001). Thus, as VEGF is a classical paracrine factor, released by one cell and acting on an adjacent

endothelial cell, systemic VEGF levels do not appear to be useful for the direct identification of patients at risk of HACE formation.

Anti-VEGF treatment blocks vascular leakage

VEGF has been considered as a causative agent in HACE formation, although no direct data were presented (Xu and Severinghaus, 1998). Our results now show that there is a direct causative relation between inhibition of VEGF action and the prevention of hypoxia-induced vascular leakage. Further indirect evidence is coming from a number of studies demonstrating a correlation between increased VEGF expression and oedema formation in brain tumours (Machein and Plate, 2000) and brain injury (Nag *et al*., 1997; Papavassiliou *et al*., 1997) as well as in cerebral ischaemia (Zhang *et al*., 2000). Inhibition of VEGF action has been considered to be useful in a variety of brain pathologies. An anti-VEGF strategy is currently being used in various clinical trials to treat tumour patients with the aim of inhibiting tumour-induced angiogenesis, thereby depriving the tumour cells of nutrients and oxygen (Carmeliet and Jain, 2000). Anti-VEGF treatment may have, in addition, a beneficial effect in these patients by reducing tumour-induced oedema formation. Furthermore, during stroke, oedema formation occurring around infarcted brain tissue was significantly reduced by the fusion protein mFlt(1-3)–IgG, which sequesters VEGF (van Bruggen *et al*., 1999). Recent data demonstrated that Src family kinases are involved in the VEGF-mediated augmentation of vascular permeability (Eliceiri *et al*., 1999). Blockade of Src activity in mice provided cerebral protection following stroke due to reduced brain oedema formation (Paul *et al*., 2001). Our results are in line with these reports and now suggest that blocking VEGF action may be a more specific therapeutic approach to the treatment of HACE. This notion is further sustained by the recent finding that dexamethasone, which is widely used at high altitude as well as in the clinic to reduce cerebral oedema, is capable of inhibiting hypoxia-induced VEGF gene expression (Heiss *et al*., 1996; Fischer *et al*., 2001).

Putative mechanisms involved in VEGF-mediated vascular permeability

It has been shown both *in vitro* (Fischer *et al*., 1999) and *in vivo* (Mayhan, 1999) that the VEGF-mediated increase in permeability of the BBB involves nitric oxide- and cGMP (cyclic guanosine monophosphate)-dependent pathways. It remains unclear, however, where these molecules exert their action. It has been hypothesized that VEGF may induce the opening of interendothelial tight junctions or change the endothelial cell phenotype, or even alter pinocytic transport through the endothelial cell (Mayhan, 2001). On the one hand, topical administration or intradermal injection of VEGF can transform continuous endothelium into fenestrated endothelium and thereby increase vascular permeability (Roberts and Palade, 1995). On the other hand, BBB permeability may be controlled by phosphorylation of tight-junction proteins (Papadopoulos *et al*., 2001). For example, occludin and zonula occludens 1 (ZO-1), which are both important constituents of the tight junction, are rapidly phosphorylated by VEGF treatment (Antonetti *et al*., 1999). Furthermore, VEGF reduced occludin expression and disrupted both ZO-1 and occludin organization at the tight junction (Wang *et al*., 2001). In an *in vitro* model of the BBB, we have recently shown that hypoxia-induced hyperpermeability involves changes in the expression of ZO-1 and that these changes are mediated by VEGF (Fischer *et al*., 2002). Taken together, these results suggest that alteration of tight-junction protein expression and phosphorylation may regulate vascular permeability.

Future research, including the generation and analysis of mice with inducible overexpression of VEGF under the control of brain-specific promoters, will help us to understand fully the mechanisms whereby VEGF induces permeability and to unravel the different effects of VEGF in the CNS involving angiogenesis, permeability induction and neuroprotection. In summary, we have shown that hypoxia-induced VEGF expression in the brain is responsible for vascular leakage. These findings provide the background for the development of therapeutic strategies to treat HACE based on the inhibition of VEGF action.

Acknowledgements

This work was supported by a grant from the Hartmann Müller-Stiftung, the Stiftung für wissenschaftliche Forschung an der Universität Zürich and the Kerckhoff Klinik Bad Nauheim. We wish to thank M. Euler for technical assistance and Lara Ogunshola and Roland H. Wenger for critically reading the manuscript.

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Received March 5, 2002. Revised June 1, 2002.
Accepted June 4, 2002